INFECTION AND IMMUNITY, Mar. 1977, p. 760-764

Vol. 15, No. 3
Printed in U.S.A.

Effect of Protein A on Staphylococcal Opsonization

PHILLIP K. PETERSON,* JAN VERHOEF, L. D. SABATH, AND PAUL G. QUIE

Departments of Medicine and Pediatrics, University of Minnesota School of Medicine, Minneapolis, Minnesota 55455

Received for publication 2 September 1976

To study the effect of cell wall protein A on bacterial opsonization, phagocytosis of 10 strains of *Staphylococcus aureus* with high and low protein A contents was measured. Those strains that contained the highest concentrations of protein A were phagocytized by human neutrophils at a slower rate than strains with little or no protein A when normal human serum and purified immunoglobulin G (IgG) were used as opsonic sources. When IgG-deficient serum was used as an opsonic source, however, protein A-rich strains were phagocytized more rapidly than protein A-deficient strains. Extracellular (purified) protein A decreased the opsonic activity of all sera tested including IgG-deficient serum. It is proposed that when IgG is not present in the opsonic medium, cell wall protein A is capable of activating complement at the bacterial surface and thereby opsonization is promoted.

Protein A, a component of the cell wall of over 90% of *Staphylococcus aureus* strains (3, 4), possesses the unique capacity of combining with the Fc fragment of immunoglobulin G (IgG) (7). Protein A-IgG complexes have been found capable of activating both the classical and alternative pathways of complement (11, 12). Dossett et al. (1) and Forsgren and Quie (6) have demonstrated that protein A is antiphagocytic in vitro, a finding consistent with protein A's anticomplementary activity and with the binding of protein A to the Fc portion of IgG, thereby interfering with bacterial attachment to the Fc receptors of phagocytic cells.

To further investigate the influence of cell wall protein A on phagocytosis, 10 *S. aureus* strains with different protein A contents were opsonized in a variety of sera, and phagocytosis by human neutrophils was measured. Since protein A is released from bacterial cell walls, and it may thereby affect humoral factors, the influence of purified protein A on serum opsonic activity was also studied.

MATERIALS AND METHODS

Bacterial strains. *S. aureus* 502A, Wood 46, and five strains isolated from patients with staphylococcal infections were studied. *S. aureus* Cowan I and two protein A-deficient mutants of this strain, NG 316 and EMS 252 (5), were kindly provided by A. Forsgren and K. Nordström (University of Malmö and Uppsala, Sweden). The NG 316 mutant produced nuclease, coagulase, alpha-hemolysin, fibrinolysin, and fermented mannitol and had the same phage type as the parent Cowan I strain. Strain EMS 252 was non-phage typable and did not produce any of the above enzymes.

Quantitation of cell wall protein A. An 18-h culture in 10 ml of Trypticase soy broth (Difco, Detroit, Mich.) of each bacterial strain was centrifuged at 1,600 × *g* for 15 min. All bacterial pellets were resuspended in phosphate-buffered saline, pH 7.4 (PBS) and adjusted to the same optical density (10% transmission at 620/pm). The suspensions were then centrifuged for 15 min and the pellets were resuspended in 2 ml of PBS containing 25 μg of lysostaphin per ml (Schwarz/Mann, Orangeburg, N.Y.) (10). After 2 h of incubation at 37°C, the transmission of light was increased at least eightfold in all bacterial suspensions. The lysate was centrifuged at 1,600 × *g* for 15 min. The content of protein A in the supernatant was determined qualitatively by double diffusion in agar (9) and quantitatively by an indirect hemagglutination method in which sheep erythrocytes sensitized with anti-sheep erythrocyte serum were incubated in tubes containing serial dilutions of the individual supernatants (13). The titer of protein A was expressed as the reciprocal of the last dilution in which there was visible hemagglutination.

Radioactive labeling. One-tenth milliliter of an overnight culture of bacteria of each strain was inoculated into 10 ml of Mueller-Hinton broth (Difco, Detroit, Mich.) containing 0.02 mCi of [*methyl*-^{3}H] thymidine (specific activity, 6.7 Ci/mmol, New England Nuclear, Boston, Mass.). After 18 h of growth at 37°C, the bacteria were washed three times in PBS. A final bacterial concentration of 5 × 10^8 colony-forming units of PBS per ml was obtained spectrophotometrically and confirmed by pour-plate colony counts.

Leukocytes. Ten to twenty milliliters of blood was drawn from healthy donors in a syringe containing 200 U of heparin. The erythrocytes were sedimented for 1 h in 6% dextran "70" (Cutter Laboratories, Berkeley, Calif.) in normal saline (10 ml of blood-3 ml of saline). The leukocyte-rich plasma was

withdrawn and centrifuged at 160 *g* for 5 min. The resulting pellet was washed twice in heparinized saline (10 U of heparin/10 ml of saline). Using a standard counting procedure, the final leukocyte suspension was made up to contain 10^7 polymorphonuclear (PMN) leukocytes/ml of Hanks balanced salt solution with 1% gelatin (HBSS).

Opsonins. Fresh human serum was pooled from five normal donors. IgG-deficient serum, in which IgG, IgA, and IgE were nondetectable by Mancini radial-immunodiffusion and Ouchterlony methods and in which IgM was within normal limits (39 mg/100 ml), was obtained from a patient with congenital rubella infection. All sera were kept frozen in aliquots at −70°C, and shortly before use were thawed and diluted in HBSS to a final concentration of 10%. Heat-inactivated sera were prepared by heating aliquots at 56°C for 1 h. Purified human IgG (kindly provided by Ralph C. Williams, Jr., University of New Mexico, Alburquerque) was stored at 4°C and used in a concentration of 300 mg/100 ml in HBSS. In one series of experiments sera were diluted in HBSS containing 100 μg of purified protein A (kindly provided by A. Forsgren) per ml of HBSS. After incubation for 30 min at 37°C, these sera, as well as the control sera, were centrifuged at 1,600 × *g* for 15 min, and the supernatants were used as opsonic sources.

Phagocytosis mixtures. Mixtures of 1.5 ml containing leukocyte suspension, opsonin, and bacteria were prepared in plastic tubes (12 by 75 mm, Falcon, Oxnard, Calif.) in a volume ratio of 5:4:1. The final bacteria-PMN leukocyte ratio was 10:1. The mixtures were tumbled at 10 rpm in a rotating rack (Fisher Roto-Rack, Fisher Scientific Co., Chicago, Ill.) at 37°C.

Sampling of the phagocytosis mixtures. To determine the leukocyte-associated counts per minute (cpm), duplicate 100-μl samples were taken from the phagocytosis mixtures with an Eppendorf pipette after 3, 10, and 20 min of incubation, placed in 3 ml of PBS in polypropylene vials (Bio-Vials, Beckman, Chicago, Ill.) and then centrifuged at 160 × *g*. The pellets were washed twice with PBS. The final leukocyte pellets were disrupted with 2.5 ml of distilled water, and after centrifuging at 1,600 × *g*, the pellets were resuspended in 2.5 ml of scintillation liquid (toluene containing fluor-alloy [TLA, Beckman] and 20% Biosolve-3 [Beckman]) and counted in a liquid scintillation counter (Beckman LS-250). To determine the total bacterial cpm (phagocytized and extracellular bacteria), duplicate 100-μl samples were taken at the end of the assay period, placed in 2.5 ml of distilled water, and centrifuged at 1,600 × *g* for 15 min. The pellets were resuspended in 2.5 ml of scintillation liquid and counted. Chemical quenching was similar for all samples.

Calculations. Averages of duplicate values, 90% of which were within 10% of agreement, were used in all calculations. The percentage of the total bacterial population which was phagocytized at a given sampling time was calculated using the formula:

$$\%\ \text{of bacteria phagocytized} = \frac{\text{cpm in leukocyte pellet}}{\text{cpm in total bacterial pellet}} \times 100.$$

RESULTS

Phagocytosis of *S. aureus* strains containing high and low concentrations of cell wall protein A. Four *S. aureus* strains with high levels of protein A (rich) and four strains possessing low levels of protein A (poor) were added in equal ratios to PMN leukocytes in the presence of normal serum. The protein A titer in the rich groups varied from 640 to 2,560, that of the poor group varied from <10 to 80. *S. aureus* Cowan I and two protein A-deficient mutants of this strain (EMS 252 and NG 316) were studied in a similar manner. The EMS 252 mutant contained no detectable protein A. The protein A titer of NG 316 was 10 compared with 640 for the parent strain. The kinetics of phagocytosis of these strains are shown in Fig. 1. The protein A-rich strains and the parent Cowan I strain were phagocytized at a slower rate than were the strains deficient in protein A. After 20 min of incubation with leukocytes, 78 to 86% of the protein A-poor bacteria were phagocytized, compared with 62 to 78% of the protein A-rich bacteria (Fig. 1A). Likewise, both mutant strains were somewhat better phagocytized than the parent Cowan I strain (Fig. 1B).

When purified IgG was used as an opsonic source, there was a marked difference in phagocytosis between the parent Cowan I strain and the two mutants. After 20 min of incubation with PMN leukocytes, only 40% of Cowan I bacteria were phagocytized compared with approximately 90% of bacteria belonging to both mutant strains (Fig. 2). When heat-inactivated normal serum was used as an opsonic source, phagocytosis of the four protein A-rich and four protein A-poor strains was less than 50% of that when normal serum was used; however, phagocytosis of the protein A-rich strains opsonized with heat-inactivated serum was lower than that of the protein A-poor strains (data not presented).

When IgG-deficient serum was used as an opsonic source, however, phagocytosis of the protein A-rich strains and of the parent Cowan I strain was greater than that of the protein A-deficient strains (Fig. 3). Whereas 63 to 69% of the protein A-rich bacteria were phagocytized after 20 min, only 14 to 61% of the protein A-poor bacteria were phagocytized (Fig. 3A). Only 21% of the mutant EMS 252 were phagocytized compared with 89% of the parent strain (Fig. 3B). There was better phagocytosis of mutant NG 316 which contained some protein A; however, there was significantly slower uptake than that of the parent strain. Heat-inactivation of IgG-deficient serum significantly lowered the opsonic capacity of this serum for the protein A-rich bacteria to a level lower than

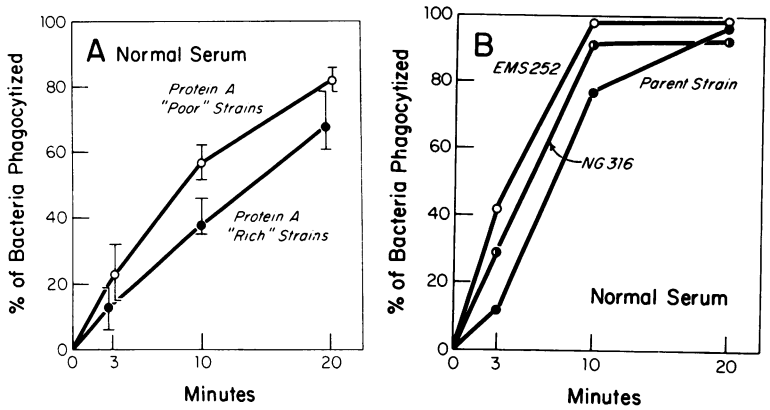

FIG. 1. *Phagocytosis of S. aureus strains in normal serum. Four S. aureus strains containing high levels of protein A (rich) and four strains containing low levels of protein A (poor) were added to PMN leukocytes in the presence of normal serum (A). S. aureus Cowan I (parent strain) and two protein A-deficient mutants (NG 316 and EMS 252) were likewise added to PMN leukocytes (B). Bars represent the extremes for the four protein A-rich and four protein A-poor strains.*

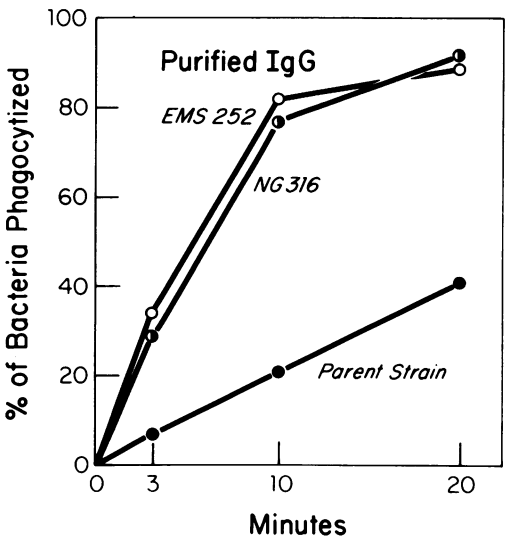

FIG. 2. *Phagocytosis of S. Aureus Cowan I (parent strain) and two protein A-deficient mutants in purified IgG.*

that of heat-inactivated normal serum (data not shown).

Influence of extracellular protein A on opsonization. The effect of soluble protein A on the opsonic activity of normal serum, heat-inactivated serum, and IgG-deficient serum was studied by incubating each of these sera with purified protein A before adding bacteria and leukocytes and measuring phagocytosis. The opsonic capacity of all three of these sera was significantly reduced after treatment with protein A (Fig. 4). Whereas 67 to 70% of bacteria were phagocytized after 20 min of incubation with leukocytes in normal serum and IgG-deficient serum, only 43 to 45% of bacteria were phagocytized in normal serum and IgG-deficient serum treated with protein A. A similar decrease in opsonic activity was found when heat-inactivated serum was incubated with protein A. These results were confirmed in experiments performed on 3 separate days with three different strains. In all experiments, incubation with protein A reduced the opsonic capacity of the different sera by greater than 40%. The decrease in phagocytosis mediated by protein A did not appear to be related to a direct effect of protein A on the phagocytic function of PMN leukocytes, as leukocytes incubated with protein A for 2 h at 37°C did not behave differently from control leukocytes.

DISCUSSION

The rate of phagocytosis of 10 *S. aureus* strains was found to correlate with the protein A content of the strains. The effect of protein A on phagocytosis depended on the opsonin used in the phagocytosis mixture. When normal serum was used as an opsonic source, those strains possessing high levels of protein A were phagocytized more slowly than strains with low levels of protein A. When purified IgG was used as an opsonic source, the protein A-rich Cowan I strain was poorly phagocytized when compared with the phagocytosis of two protein A-deficient mutants of this strain. Likewise, although heat-inactivated serum was not as good an opsonic source as was normal serum for all the strains, the protein A-rich strains were phagocytized more slowly than were the protein A-poor strains when heat-inactivated se-

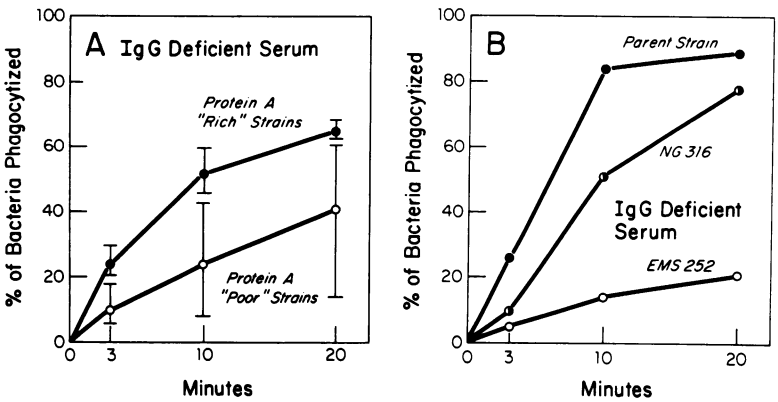

FIG. 3. *Phagocytosis of S. aureus strains in IgG-deficient serum. Four S. aureus strains containing high levels of protein A (rich) and four strains containing low levels of protein A (poor) were added to PMN leukocytes in the presence of IgG-deficient serum (A). S. aureus Cowan I (parent strain) and two protein A-deficient mutants were likewise added to PMN leukocytes (B). Bars represent the extremes for the four protein A-rich and four protein A-poor strains.*

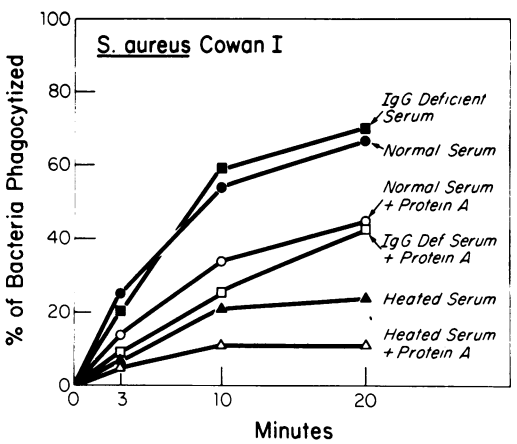

FIG. 4. *Effect of purified protein A on the opsonic activity of normal serum, IgG-deficient serum and heat-inactivated serum. Normal serum was incubated with HBSS (●) and HBSS containing 100 μg of protein A (○); IgG-deficient serum was incubated with HBSS (■) and HBSS containing 100 μg of protein A (□); heat-inactivated serum was incubated with HBSS (▲) and HBSS containing 100 μg of protein A (△). After centrifuging, the supernatants were used as opsonic sources in phagocytosis mixtures containing S. aureus Cowan I and PMN leukocytes.*

rum was used as an opsonin. In contrast to these findings, when IgG-deficient serum was used as an opsonic source, the protein A-rich strains were more rapidly phagocytized than were the protein A-deficient strains. Thus, it appeared that cell wall protein A had the capacity to inhibit opsonization when IgG was present in the opsonic medium but paradoxically enhanced opsonization in the absence of IgG.

When purified protein A in a concentration of 100 μg/ml was incubated with normal serum, heat-inactivated serum, and IgG-deficient serum, the opsonic activity of all three sera was significantly reduced. This inhibitory effect on the opsonic capacity of normal serum is in keeping with the findings of Dossett et al. (1) and Forsgren and Quie (6). As pointed out by these investigators and as evidenced by Stålenheim et al. (12), this inhibition is most probably mediated through the binding of protein A to the Fc fragment of IgG, as well as through the activation of complement mediated by IgG-protein A complexes. The inhibitory effect of protein A on the opsonic activity of heat-inactivated serum is presumably mediated through the blocking of Fc fragments alone.

As a similar inhibitory effect was found when purified protein A was incubated with IgG-deficient serum, it appeared that protein A was also capable of activating complement in the absence of IgG-protein A complex formation. The IgG-deficient serum used in these experiments did contain IgM. Grov (8) has recently shown that protein A is capable of binding to IgM in the region of its Fc fragment. It is therefore possible that IgM-protein A complexes were formed in the IgG-deficient serum and that such complexes activated complement. However, IgG-deficient serum which in addition contained very low levels of IgM has been found capable of opsonizing protein A-rich staphylococcal strains, but not protein A-poor strains (J. Verhoef, submitted for publication). Therefore, it seems likely that protein A is capable of activating complement in the absence of immunoglobulin. Additional studies

will be necessary to determine the minimal concentration of protein A that will produce an inhibitory effect on the opsonic capacity of IgG-deficient serum and to quantitate the degree of complement activation.

Thus, the effect of protein A on opsonization of *S. aureus* depends not only on the location of this protein (in the bacterial cell wall or an extracellular locus) but also on the opsonic source being studied. When IgG is present, both cell wall and extracellular protein A inhibit opsonization of bacteria. When IgG is not present, however, cell wall protein A appears to be capable of activating complement at the bacterial surface and thereby actually promotes effective bacterial opsonization. When it is acting extracellularly, protein A inhibits opsonization by IgG-deficient serum by activating complement in the medium so that it is no longer available for activation at the baterial surface.

There is reason to believe from such in vitro data that protein A may play a role in the severity of staphylococcal infection. If a staphylococcal infection becomes established, organisms replicate and protein A is released from the staphylococcus, which can then potentially inactivate complement as well as block the Fc fragments of IgG. Cell wall protein A may either inhibit or enhance phagocytosis depending on the concentration of IgG present.

ACKNOWLEDGMENTS

The laboratory assistance of Hattie Gray and Marge Lindemann and the secretarial help of Shirley Hermel were greatly appreciated.

This work was supported in part by Public Health Service grants AI06931 and AT08821 from the National Institute of Allergy and Infectious Diseases. J. Verhoef was supported ty the Netherlands Organization for the Advancement of Pure Research, and P. Peterson is a recipient of a Bristol Research fellowship in Infectious Diseases.

LITERATURE CITED

1. **Dossett, J. H., G. Kronvall, R. C. Williams, Jr., and P. G. Quie.** 1969. Antiphagocytic effects of staphylococcal protein A. J. Immunol. **103**:1405–1410.
2. **Forsgren, A.** 1969. Protein A from *Staphylococcus aureus*. VIII. Production of protein A by bacterial and L-forms of *Staphylococcus aureus*. Acta Pathol. Microbiol. Scand. **75**:481–490.
3. **Forsgren, A.** 1970. Significance of protein A production by staphylococci. Infect. Immun. **2**:672–673.
4. **Forsgren, A., and K. Nordstrom.** 1974. Protein A from *Staphylococcus aureus:* the biological significance of its reaction with IgG. Ann. N.Y. Acad. Sci. **236**:252–266.
5. **Forsgren, A., K. Nordstrom, L. Philipson, and J. Sjöquist.** 1971. Protein A mutants of *Staphylococcus aureus*. J. Bacteriol. **107**:245–250.
6. **Forsgren, A., and P. G. Quie.** 1974. Effects of staphylococcal protein A on heat-labile opsonins. J. Immunol. **112**:1177–1180.
7. **Forsgren, A., and J. Sjöquist.** 1966. Protein A from *S. aureus* I. Pseudoimmune reactions with human γ-globulin. J. Immunol. **97**:882–827.
8. **Grov, A.** 1975. Human IgM interacting with staphylococcal protein A. Acta Pathol. Microbiol. Scand. **83**:173–176.
9. **Lind, I., I. Live, and B. Mansa.** 1970. Variation in staphylococcal protein A reactivity with γ-globulins of different species. Acta Pathol. Microbiol. Scand. **78**:673–682.
10. **Sjöquist, J., B. Meloun, and H. Hjelm.** 1972. Protein A isolated from *Staphylococcus aureus* after digestion with lysostaphin. Eur. J. Biochem. **29**:572–578.
11. **Sjöquist, J., and G. Stålenheim.** 1969. Protein A from *Staphylococcus aureus*. IX. Complement-fixing activity of protein A-IgG complexes. J. Immunol. **103**:467–473.
12. **Stålenheim, G., O. Götze, N. R. Cooper, J. Sjöquist, and H. J. Müller-Eberhard.** 1973. Consumption of human complement components by complexes of IgG with protein A of *Staphylococcus aureus*. Immunochemistry **10**:501–507.
13. **Winblad, S., and C. Ericson.** 1973. Sensitized sheep red cells as a reactant for *Staphylococcus aureus* protein A. Acta Pathol. Microbiol. Scand. Sect. **81**:150–156.